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TITLE: EphA2 Kinase Agonists as Novel Suppressors of Both Prostate Cancer Cell Motility and Growth: Structure-Function Relations and the Role of RAS/MAPK Pathway

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13. ABSTRACT (Maximum 200 Words) There are three goals in this proposal. 1) To establish the expression profile of EphA2 during TRAMP prostate cancer development and progression, which will help determine the optimal window of EphA2 targeting. 2) To target EphA2 with its native and artificial agonists to see if they can suppress TRAMP prostate cancer development and/or progression. 3) To investigate the role of EphA2 during prostate cancer development by crossing EphA2-null mice with TRAMP mice. During the past 6 months since this modified grant was reactivated, we have made significant progress breeding and genotyping large number of animals required for achieving the goals. FVB/TRAMP F1 mice are ready long-term histopathological characterization of EphA2 expression. The backcross of EphA2-/- mice to pure C57B1/6, necessary for achieving the third goal has also reached F3. We are currently using these animals to test the hypothesis that EphA2 kinase is involved in the prostate cancer development and progression and can be target in the treatment and prevention of the disease.				
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A. INTRODUCTION

A1 Scientific and Administrative Changes to the Original Proposal Affecting this Annual Report

Since last annual report, significant changes have taken place with this Phase II New Investigator Grant. As the PI reported to USAMRMC in last year's annual report, a similar grant that the PI had submitted to NIH was funded soon after this Phase II grant was activated. As a result, the PI stopped using the funding from this grant in April of 2002. With the permission from USAMRMC, we started modifying the specific aims of original proposal. The modified proposal focused on a project that was closely related with the original proposal. In fact the newly proposed studies are designed to test the same hypothesis of the original proposal, but utilize more relevant model systems that have just become available. Specifically, in the original application, we proposed to use xenograft model to test the hypothesis that that EphA2 activation by its agonists can inhibit prostate cancer growth and progression. In the modified proposal, genetically engineered mouse model will be used to test the same hypothesis. The request to modify the original proposal was approved by USAMRMC in March 2003, and the grant was reactivated in April 2003. Therefore this particular annual report will cover recent progress in the modified proposal in the last six months.

A2 General Introduction

For a prostate cancer patient, the difference between life and death is whether the tumor cells have metastasized or not. There are two key determinants in successful tumor metastasis. One is tumor cell dissemination to a distal organ; the other is metastatic tumor cell growth at the new site. Cell motility plays a critical role in tumor cell dissemination, both during invasion of surrounding tissues at the original tumor site, and during extravasation and migration to favorable growth sites at the distal organ. Indeed, prostate cancer cell motility in vitro has been directly correlated with metastatic potential of prostate cancer in vivo (1;2). Metastatic tumor cell growth at distal sites is regulated by paracrine and autocrine factors, many of which converge on Ras/Raf/MEK/ERK1/2 MAP kinase (MAPK) growth stimulatory signaling pathway. Recent studies have shown that MAPK activity is elevated in prostate cancer specimens compared with normal tissue (3;4), the degree of which is correlated with tumor progression (3;5). Therefore, agents that can suppress either prostate cancer cell motility or MAPK activity can potentially be exploited to prevent metastasis in vivo. Agents that can accomplish both feats will be even more desirable.

We have found that agonists of EphA2 kinase possess this unique combination of properties, i.e. they were capable of suppressing both cell motility (6) and Ras/MAPK signaling cascade in prostate cancer epithelial cells (7). EphA2 activation by its natural ligand or an agonistic peptide isolated from peptide phage display libraries inhibited cell adhesion, spreading and migration by targeting integrins and focal adhesion kinase (FAK). Unexpectedly, activated EphA2 also potentially suppressed the ERK1/2 MAPK activity. In keeping with the growth regulatory role of MAPK, activation of EphA2 kinase inhibited cell proliferation. More recently, the PI's laboratory has found that EphA2 activation on DU-145 cells could potentially counteract the stimulatory effects of hepatocyte growth factor/scatter factor-induced cell scattering, migration, matrix invasion and epithelial-mesenchymal transition similar to what we have recently reported for MDCK epithelial cells (8). Therefore, we hypothesized that EphA2 can be targeted in the prevention and treatment of prostate cancer progression. The goal of this Phase II New Investigator proposal is to test this hypothesis.

B. BODY

B1. List of Original SPECIFIC AIMS

- 1) Using TRAMP mice as a new preclinical model system, we will determine expression of EphA2 during tumor progression and the therapeutic efficacy of EphA2 agonists.
- 2) We will breed TRAMP/EphA2^{-/-} mice to investigate whether and how EphA2 may contribute to prostate cancer development and progression.

B2. PROGRESSES (Itemized according to statement of work)

General comments: There are three goals in this proposal. 1) To establish the expression profile of EphA2 during TRAMP prostate cancer development and progression, which will help determine the optimal window of EphA2 targeting. 2) To target EphA2 with its native and artificial agonists to see if they can suppress TRAMP prostate cancer development and/or progression. 3) To investigate the role of EphA2 during prostate cancer development by crossing EphA2-null mice with TRAMP mice. All three goals involve large-scale breeding of transgenic and/or knockout mice. Due to the high cost associated with large-scale breeding experiments, the proposed studies were not commenced until the grant was reactivated in late April 2003. Breeding transgenic and knockout mice is time-consuming by nature, particularly when it involves backcrossing as in Task 3. During the past few months since the grant was reactivated, we have made significant progresses in breeding and genotyping large numbers of transgenic and knockout mice. We are poised to generate large cohorts of transgenic and knockout mice to test the hypothesis originally proposed.

The large-scale animal studies proposed in this modified proposal is labor- and resource-intensive. Since the modified proposal was approved without additional support, the limited resource is posing problems, although it did allow us to get started on the projects. The PI is actively seeking additional funding to continue these studies.

B2.1 TASK 1. To investigate expression of EphA2 kinase during different stages of PCa progression in TRAMP mice.*1). To breed and phenotype TRAMP mice (Months 1-4).*

Completed. We have obtained TRAMP mice from NCI. They are being bred in the lab and are routinely genotyped according the published PCR-based procedures (9). The colony is currently being expanded. Female offspring are being bred with FVB mice.

2). To breed FVB mice (Months 1-4).

Completed. Wild type FVB mice have been obtained from Jackson laboratory. The colony has been expanded. Male offspring are currently being bred with TRAMP mice.

3). To cross FVB with TRAMP mice to obtain F1 male mice (Months 5-8).

In progress. The first sets of FVB and C57Bl/6 TRAMP breeding pairs have been set up. Several litters of F1 mice have been born and will be genotyped.

4). Histopathological evaluation of EphA2 expression at different developmental stages (Months 5-12).

To be performed. F1 FVB/TRAMP male mice from above will be randomly grouped according ages and analyzed histopathologically to examine how EphA2 expression is regulated during

prostate cancer progression. The information will help us determine the appropriate window to target EphA2 with agonists.

TASK 2. To test the efficacy of EphA2 agonists on PCa development and progression in TRAMP mice.

1). To produce recombinant EphA2 agonists in large quantities for in vivo studies (Months 3-6).

We have optimized the procedures for producing recombinant ephrin-A1-Fc, which has helped us to produce 200 mg of the proteins. More is being produced and stored at -80C in preparation for the in vivo experiments.

2). To test the efficacy of EphA2 agonists in inhibiting prostate cancer progression in TRAMP mice (Months 7-12).

To be performed when a potential susceptible window is identified according to histopathological analysis in Task 1.

TASK 3. To determine how EphA2 knockout may modulate prostate cancer development in TRAMP mice.

It has come to our realization that the EphA2^{-/-} mice have been maintained on mixed C57Bl/6J x 129 mixed genetic background at the NIH-sponsored consortium where we obtained the mice. Due to the complex breeding involved in the proposed studies here, it is important to have EphA2^{-/-} mice on pure C57Bl/6 genetic background on to avoid complications in data interpretation. This part of the project was not included in the original proposal, but is necessary to carry out. Therefore, in the past 6 months, we have conducted backcross of EphA2^{-/-} on C57Bl/6J x 129 mixed genetic background to C57Bl/6. The F3 pups have been born for two weeks and F4 will be used for breeding mice used Task 3.

1). To breed TRAMP heterozygous female mice with EphA2^{-/-} male mice (months 4-8).

2). To breed TRAMP/EphA2^{+/-} mice with EphA2^{+/-} mice to generate the following phenotypes: TRAMP/EphA2^{+/+}, TRAMP/EphA2^{+/-}, and TRAMP/EphA2^{-/-}. (Months 8-12).

3). To breed TRAMP/EphA2^{+/-} mice with EphA2^{+/-} mice to generate the following phenotypes: TRAMP/EphA2^{+/+}, TRAMP/EphA2^{+/-}, and TRAMP/EphA2^{-/-}. (Months 8-12).

4). To breed TRAMP/EphA2^{+/-} mice with EphA2^{+/-} mice to generate the following phenotypes: TRAMP/EphA2^{+/+}, TRAMP/EphA2^{+/-}, and TRAMP/EphA2^{-/-}. (Months 8-12).

These Tasks will be performed once the F4 backcross is completed as described in the last paragraph.

C. KEY RESEARCH ACCOMPLISHMENTS

- Obtained EphA2^{-/-} mice backcrossed to C57Bl/6 background, which will be valuable not only for the proposed studies but also in other areas of studies on the role of Eph kinases in tumor development and progression.
- Generated FVB x C57Bl/6 F1 TRAMP mice.
- Designed a novel strategy to purify recombinant ephrin-A1 ectodomain produced in mammalian cells.
- Purified large quantities of fusion proteins for in vivo therapeutic studies.

D. REPORTABLE OUTCOMES

1) Promotion based on the studies funded by DOD

- Dr. Hui Miao, a key personnel on Phase I and Phase II, has been promoted to faculty at Case Western Reserve University School of Medicine as an Instructor.

2) Funding obtained based on the studies funded by DOD:

- As a direct result of DOD funding, the PI has received a Prostate Cancer Foundation Award for 2004.

3) Publications:

- Myshkin, E. and Wang, B. (2003). Chemometrical classification of ephrin ligands and Eph kinases using GRID/CPCA approach. **Journal of Chemical Information and Computer Sciences** 43:1004-1010.
- Deroanne, C., Valérie Vouret-Craviari, V., Wang, B. and Pouyssegur J. (2003). EphrinA1 inactivates integrin-mediated vascular smooth muscle cell spreading via the Rac/PAK pathway. **Journal of Cell Sciences** 116:1367-1376.
- Miao, H., Nickel, C., Cantley, L. G., Bernaddo, L., Bruggeman, L. and Wang, B. (2003) EphA kinase activation regulates epithelial branching morphogenesis. **Journal of Cell Biology** 162:1281-1292.

4) Manuscript:

- Miao, H., Guan, J.-L., Shen, T. L., Strebhardt, K., Paquale, E. B., and Wang, B. Inhibition of integrin-mediated cell adhesion and spreading, but not migration requires the catalytic activity of EphB3 kinase. **Journal of Biological Chemistry** (*under revision*).
- Wei, B.-R. Miao, H. and Wang, B. Ephrin-A1 stimulation of endogenous EphA kinases inhibits prostate cancer cell chemotaxis and invasion. **Cancer Research** (*Submitted*).

E. CONCLUSION

In sum, over the short few months since this Phase II New Investigator Grant was reactivated, we have made significant progress in the key breeding experiments. Significant cohorts of transgenic and knockout mice have been bred. The EphA2^{-/-} mice backcross to C57Bl/6 is on track to useable in the next months. We now poised to set up large scale group for performing the proposed studies. Finally, a strong evidence of accomplishment of these DOD funded projects is the fact that it has led to significant federal funding to the PI from NCI that has replaced the original Phase II proposal, as well as the new funding from the Prostate Cancer Foundation (formerly CaP CURE). This will ensure continued long-term success of these studies. It is noteworthy that frequent mutations of Eph kinases have recently been reported in human prostate cancer by J. Milbrandt. The extensive in vivo

experiments ongoing in the PI's lab, albeit still in early stage, will address the critical questions on the role of Eph kinases in prostate cancer development and progression. They will also provide a key preclinical model system to test if Eph kinases can be targeted for prostate cancer therapy.

However, one problem that is becoming evident with the current modified proposal is the very limited resources available to complete all the studies, which involves large-scale breeding, long-term caging, and sophisticated analyses of transgenic and knockout mice. The PI is actively seeking additional funding to continue the proposed studies.

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Appendix 1

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X-Mailer: Internet Mail Service (5.5.2656.59)
Original-recipient: rfc822;bxw14@po.cwru.edu

Dear Dr. Wang,

Dr. Mishra has forwarded your paperwork requesting a extension without funds and a change in the SOW to me. I am the Contract Specialist assigned your research grant.

We will allow you a one year no cost extension. A modification will be forthcoming. Also, we will allow the change in your SOW. This will be included in the mod for the extension.

However, you will need to contact our Animal Use department to get their approval for your use of mice. The POC is Joyce O'Brien 301-619-2144. Please contact her ASAP so this will not delay your award. I will **not** be doing the mod for the extension without funds until I receive the animal use approval from Ms. O'Brien, that way I only have to do one modification.

If you have any further questions, don't hesitate to contact me.

Sherrie Walters
Contract Specialist
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Appendix 2

Stimulation of EphA Kinases on Prostate Adenocarcinoma Cells Antagonizes c-Met-Mediated Chemotaxis and Invasion

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Key Words: Eph, ephrin, prostate cancer, HGF/SF, E-cadherin

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ABSTRACT

Expression of Eph kinases and their ephrin ligands has been documented in various cancer cells in vitro and in vivo. However, the biological function of individual Eph kinases in these cells remains unclear. This is complicated by the promiscuity of Eph/ephrin interactions, necessitating assessment of overall receptor and ligand expression. Here, we show that EphA2 is the predominant EphA kinase expressed on human and rat prostate cancer cell lines. Moreover, expression of EphA2, but not that of EphA1, EphA3 and EphA4, is correlated with metastatic potential of tumors from which the cells were derived. In contrast, the expression of ephrin-As remains low and relatively constant. Data mining of the curated microarray datasets revealed that EphA2 was significantly overexpressed during progression from localized to metastatic human prostate cancer specimens compared with localized ones. Interestingly, cell lines expressing high levels of EphA2 also overexpress c-Met, a receptor for hepatocyte growth factor/scatter factor (HGF/SF). Since the HGF/SF-c-Met axis is implicated in malignant progression of prostate cancer, we investigated potential cross talk between EphA2 and c-Met in DU-145 cells that overexpress both receptors. Ephrin-A1 stimulation significantly suppressed HGF/SF-induced chemotaxis as well as MatriGel invasion. Moreover, HGF/SF-induced cell scattering and chemokinetic motility were inhibited by ephrin-A1, which was associated with a strikingly preservation of E-cadherin localization at cell-cell junction. Our results show that EphA2 activation can counteract the stimulatory effects of HGF/SF-c-Met paracrine loop on malignant behaviors of prostate cancer cells, suggesting that EphA2 can be targeted to curb prostate cancer progression.

INTRODUCTION:

Prostate cancer is the second leading cause of cancer death for North American men, and is known to exhibit widely variable aggressiveness. While the majority (80%) of 220,000 newly diagnosed prostate cancer cases each year will remain dormant and do not metastasize during the lifetime of the patients, over 30,000 of them will rapidly metastasize and kill the patients. The molecular mechanisms governing the slow, non-threatening progression versus the rapid emergence of metastasizing lethal disease is unclear. However, for prostate cancer cells, there is a direct correlation between motility in vitro and metastatic potential in vivo ¹. As prostate cancer progresses, a growth factor autocrine/paracrine loop is often developed to promote the growth and migration of tumor cells as they acquire androgen independency and metastatic potential. The ability to block cytokine-induced prostate cancer cell migration will potentially render these cells stationary and non-metastatic ².

HGF/SF is among the growth factors linked to prostate cancer autocrine/paracrine progression ³. In normal prostate, HGF/SF is secreted by stromal cells and binds to protein tyrosine kinase receptor c-Met expressed on epithelial cells. The aberrant expression of c-Met has been reported in poorly differentiated prostate cancer ⁴ and prostate cancer bone metastasis ^{5,6}, while high level of HGF/SF expression is detected in bone marrow stroma ⁷, suggesting a possible role of HGF/SF-c-Met signaling in bone metastasis. The activation of c-Met sets off a plethora of cellular events, such as cell scattering, migration, and invasion, all of which play a role in tumor metastasis ⁸. Antagonizing these cellular events triggered by the activated c-Met represents an active area of research aimed at development of new therapeutic strategies for prostate cancer ⁹.

The 16 members of Eph receptors constitute the largest subfamily of receptor tyrosine kinases and bind to membrane-anchored ligands called ephrins. They are divided into EphA and EphB kinases according to ligand binding specificity and sequence homology. While EphA kinases bind to GPI-anchored ephrin-As, EphB kinases target transmembrane ephrin-Bs. Our recent studies using molecular modeling show that sequences in the ligand-binding domains alone were sufficient to allow classification of Eph kinases into A and B subfamilies ¹⁰. Eph receptors participate in cellular events involved in embryonic development, including axon path finding, organ cell boundary setting and angiogenesis ¹¹⁻¹³. In addition to their functions in normal tissues, abnormal expression of Eph receptors and/or their ligands, ephrins, have been reported in various cancers (see ref ¹⁴ for review). In prostate cancer, the expression of EphA2 is reportedly up-regulated ¹⁵. The significance of this overexpression in prostate cancer progression is yet to be determined. This is further complicated by the promiscuity of Eph kinase/ephrin ligand interactions, in that one receptor can interact with multiple ligands and vice versa. As a result, an estimation on the overall expression levels of both receptors and ligands on a given cell type is necessary to attribute biological response to a specific Eph kinase.

Here we report that EphA2 is the predominant EphA kinase expressed on prostate cancer cell lines. Moreover, expression of EphA2 but not that of other EphA kinases is elevated in cells derived from more metastatic prostate cancer. On the other hand, expression of ephrin-As remains low and relatively constant during tumor progression, which may account for the very low basal activation of EphA2 kinase even in cells that express high levels of the receptor. More importantly, stimulation of the endogenous EphA2 on DU-145 cells that are known to express high level c-Met could inhibit HGF/SF-induced chemotaxis, invasion and scattering. Cellular and biochemical evidence suggests that preservation of E-cadherin mediated cell-cell adhesion

is one mechanism responsible for the inhibitory effects on cell scattering. Since loss of cell-cell adhesion is a hallmark of malignant prostate cancer, EphA2 may be targeted to suppress prostate cancer progression promoted by the paracrine/autocrine growth factors.

MATERIALS AND METHODS

Cells and Reagents

CWR22R, PC-3, and LNCaP cells were cultured in RPMI medium (GIBCO) supplemented with 0.29 mg/ml glutamine, 100 µg/ml streptomycin, 100 units/ml penicillin (GIBCO), and 10% fetal bovine serum (HyClone). DU-145 cells and rat prostate cancer cells (G, AT-1, MatLu, and MatLyLu) were maintained in DMEM medium containing same supplements as in RPMI medium. HGF/SF, and goat anti-EphA1, EphA4, ephrin-A1, ephrin-A2, and ephrin-A4 were purchased from R&D Systems. Fc and Ephrin-A1-Fc were prepared as previously described^{16,17}. Rabbit anti-EphA2, rabbit anti-human c-Met, mouse anti-phosphotyrosine (PY99), mouse anti-phospho ERK (p-ERK), rabbit anti-ERK and horse radish peroxidase (HRP)- conjugated goat anti-mouse IgG were purchased from Santa Cruz. Mouse anti-E-cadherin was purchased from Transduction Laboratory. Rabbit anti-EphA3 was a gift from Dr. E. Pasquale. HRP-conjugated Protein A, Texas Red-conjugated goat anti-rabbit IgG and FITC-conjugated rabbit anti-mouse IgG were from Jackson ImmunoResearch.

Immunofluorescence

Cells were cultured on cover slips in 24-well dishes for 24 hours, fixed in 4% formaldehyde for 20 minutes and permeabilized with 0.5% NP-40 for 10 minutes. Blocking was performed using blocking solution containing 2% BSA and 2% goat serum in PBS. Subsequently, cells were incubated with 1 µg/ml anti-EphA2 or anti-E-cadherin antibody. A Texas Red-conjugated

goat anti-rabbit IgG or FITC-conjugated rabbit anti-mouse IgG was used to visualize the primary antibodies. Vector-Shield mounting solution containing DAPI (Vector Laboratories) was used to mount the cover slips onto slides.

Cell stimulation, immunoprecipitation, and immunoblotting

Cells cultured in 6 well plates at 70 - 80% confluence were stimulated with 0.5 $\mu\text{g/ml}$ Fc, 1 $\mu\text{g/ml}$ ephrinA1-Fc or 20 ng/ml HGF/SF or their combinations as indicated. After stimulation, cells were lysed in RIPA buffer (20mM Tris, pH7.4, 125mM NaCl, 20mM NaF, 0.1% SDS, 10% glycerol, 0.5% sodium deoxycholate, and 1% TX-100, 0.5mM Na_3VO_4) containing protease inhibitors (1mM phenylmethylsulphonyl fluoride, and 2 $\mu\text{g/ml}$ each of aprotinin and leupeptin). Cell lysates were clarified by centrifugation at 13,000 rpm for 5 min. Immunoprecipitation was carried out by adding anti-EphA2 antibody to clarified cell lysates and incubated for 1 hour at 4°C. The immunocomplex was captured using γ -bind beads (Pharmacia). Cell lysates or precipitated proteins were separated on 4 – 20% Tris-glycine gels (Invitrogen) and transferred to Immobilon-P PVDF membranes (Millipore). Membranes were blotted according to manufacturer's suggestion. For quantitative analysis, band densities were measured using KODAK 1D image analysis software (Eastman Kodak Company).

Immunodepletion

Cell surface proteins were labeled with biotin using ImmunoPure NHS-LC-Biotin (Pierce) according to manufacturer's suggestion. Cells were lysed in RIPA buffer and an EphA2-specific antibody was used to remove EphA2 molecules from cell lysates by repeated immunoprecipitation. The specificity of the antibody was verified using EphA2 knockout mice, where EphA2 but not other EphA kinases was absent from homozygous embryonic tissues (not shown). EphrinA1, which recognized all members of EphA kinases, was then used to precipitate

the remaining EphA kinases. Avidin immunoblot was carried out to detect the presence of ephrinA1-precipitated EphA kinases in the cell lysates with or without prior EphA2 depletion.

Cell migration and invasion assay

Modified Boyden chambers of 8 μm pore size (Costar) were used for migration assay. Both sides of the membranes were coated with 10 $\mu\text{g/ml}$ collagen at 4°C overnight. Serum free medium containing 20 ng/ml HGF/SF and/or 1 $\mu\text{g/ml}$ ephrin-A1-Fc was added to the lower chamber, while 10^5 cells in 100 μl RPMI containing 0.5% BSA were placed in the upper chamber. After incubating at 37°C for 8 hours, cells were fixed in 4% paraformaldehyde and stained with crystal violet. The cells remaining in the upper chamber were removed using cotton swabs. Cells migrated to the underside of the membrane were counted under 200x magnification.

For invasion assay, plugs of 50 $\mu\text{g/ml}$ of growth factor-reduced MatriGel (BD) were dried on the upper chamber and rehydrated according to manufacturer's instructions. The same procedure as in migration assay was followed, except that the cells were allowed 24 hours to migrate to the underside of the membranes.

Scratch-induced cell migration assay

Monolayer of confluent DU-145 cells on 6-well dishes were scratched using a micropipette tip. The cells were then washed twice with culture medium and images of the scratch were collected immediately at marked area using Leica DMIRE2 microscope. Fc or ephrin-A1-Fc in the presence or absence of HGF/SF were then added to the culture medium and cells were cultured for 12 hours. Images of the same areas were taken again at the end of incubation.

Scattering onto Immobilized Ligand (SOIL) Assay

The SOIL assay that measures cell scattering and migration was performed essentially as described ¹⁶. Briefly, ephrin-A1-Fc (4 $\mu\text{g/ml}$) or Fc (2 $\mu\text{g/ml}$) was coated on 6-well culture

dishes overnight at 4°C and the wells were washed with PBS. DU-145 cells were grown on cover slips in 24-well dishes to near confluence. Cover slips were transferred cell-side-up onto the coated 6-well dishes containing 1.5 ml culture medium with or without HGF/SF. Cover slips were gently pressed down with pipette tips. After 48 hours, cells were fixed with 4% paraformaldehyde for 20 min and stained with crystal violet. Cover slips were removed, and the rings of cells that have scattered and migrated onto the immobilized Fc or ephrin-A1-Fc were photographed.

RESULTS

EphA2, but not its ephrin-A ligands, is highly expressed in metastatic prostate cancer cells of human or rat origin.

We have previously identified EphA2 as the primary EphA kinase expressed in PC-3 cells¹⁸. Immunodepletion experiment revealed that EphA2 is also the major EphA kinase in other human prostate cancer cell lines (Fig. 1A). Interestingly, cells derived from distal metastasis including PC-3¹⁹ and DU-145²⁰ expressed much higher levels of EphA2 than cells derived from primary tumor (CWR22R)²¹ or local metastasis (LNCaP)²² (Fig. 1B and C). However, because these cells were derived from different individuals, a correlative relationship between EphA2 expression and tumor progression cannot be established. Thus, we examined the expression of EphA2 in a series of rat prostate cancer cell lines derived from the same original tumor, Dunning rat tumor R-3327²³. While R-3327-G (G) is a slow growing and androgen responsive subline with low metastatic ability, AT-1 is independent of androgen but remains poorly metastatic. MatLu and MatLyLu arose from AT-1, but are highly metastatic to lung (MatLu and MatLyLu) and lymph node (MatLyLu). We found that EphA2 expression was low in G line, moderately

increased in AT-1, and greatly elevated in MatLu and MatLyLu variants (Fig. 1B), suggesting a possible correlation between EphA2 expression and tumor progression. In contrast to EphA2, other EphA kinases, including EphA1, EphA3 and EphA4, showed no consistent changes during tumor progression in human or rat cell lines (Fig. 1B). Like other Eph kinases, EphA2 is promiscuous in ligand recognition and can bind to multiple ephrinA ligands. Immunoblot with a panel of antibodies against several ephrin-As showed that the ligands were expressed in low levels, and did not exhibit significant differences among the cell lines examined (Fig. 1B). In summary, while EphA2 expression is elevated in cell lines derived more metastatic cancers, ephrin-A expression remains low and relatively constant.

To investigate the *in vivo* relevance of our findings, we took advantage of existing microarray studies on gene expression during prostate cancer progression. The datasets from these studies have been curated to enable data mining at www.oncomine.com²⁴. A search of Oncomine revealed that in a comprehensive study designed to look for signature expression profile of prostate cancer at various stages²⁵, EphA2 was found to be significantly elevated ($p=0.0019$) in 23 metastatic samples when compared with 16 localized diseases. Thus both in cell lines *in vitro* and human prostate cancer *in vivo*, EphA2 becomes overexpressed during progression to metastasis.

Next, we examined ligand responsiveness and downstream signaling of EphA2 kinase in cells that overexpress it. DU-145 and MatLyLu were stimulated with ephrin-A1 dimerized by fusion to the heavy chain of human IgG1 (ephrin-A1-Fc). Similar to what we reported in PC-3 cells¹⁸, in both DU-145 and MatLyLu EphA2 activation readily occurred in two minutes after the addition of ephrin-A1-Fc and persisted for the duration of the experiment (Fig. 1D). ERK1/2 activity was reduced as EphA2 became activated, consistent with our previous report in a

number of cell lines, including PC-3 cells²⁶. Therefore, EphA2, but not other EphA kinases or its cognitive ligands, was highly expressed in metastatic prostate cancer cells; ligation of unoccupied EphA2 receptor readily transduced signals into cell interior.

EphA2 activation antagonized stimulatory effects of HGF/SF on cell migration

In prostate cancer, there is a direct correlation between cell motility in vitro and metastasis in vivo¹. Cell motility in turn is stimulated by the acquisition of paracrine/autocrine loops which frequently develop during prostate cancer progression. HGF/SF is one such cytokine that is suspected to play a critical role in both prostate cancer cell spreading from original tumor site and prostate cancer cell homing to the bones (Introduction). Interestingly, we found that EphA2 expression paralleled that of c-Met in human prostate cancer cell lines (Fig. 2A), i.e. low in LNCaP and CWR22R and high in DU-145 and PC-3, as reported previously⁵. Because HGF/SF-c-Met axis has been implicated in malignant progression of prostate cancer^{6,27}, we next investigated how activation of EphA2 overexpressed on metastatic prostate cancer cells may impact HGF/SF-stimulated cell migration and invasion. DU-145 cells were chosen for these studies because they have been frequently used as a model to assess the role of HGF/SF-c-Met axis in prostate cancer progression^{5,28}.

First we performed a chemotactic cell migration assay using modified Boyden chamber system (Methods). DU-145 cells were placed in the upper chamber while Fc, ephrin-A1-Fc, HGF/Fc, or HGF/ephrin-A1-Fc was added into the lower chamber (Fig. 2B). In comparison with Fc control, the presence of ephrin-A1-Fc in the lower chamber reduced the basal level of cell migration (columns 1 vs. 2; $p=4\times10^{-4}$). HGF/SF stimulated cell migration (columns 1 vs. 3; $p=1.5\times10^{-6}$), which was also potently inhibited by ephrin-A1-Fc (columns 3 vs. 4; $p=1.9\times10^{-5}$). Therefore, ephrin-A1 inhibited both basal and HGF/SF-induced cell migration.

The Boyden chamber assay measures the cellular response to a gradient of HGF/SF and may simulate tumor cell homing to target organs secreting chemotactic factors. On the other hand, tumor cells in original tumor site may be constantly exposed to growth factors produced by tumor cells themselves (autocrine), or surrounding stromal cells, or infiltrating leukocytes (paracrine). We tested if exogenously added ephrin-A1 ligand exhibited inhibitory effect on cell migration in a growth factor-filled environment. To this end, HGF/SF was added to both sides of chambers while Fc or ephrin-A1-Fc was added to the lower chambers only (Fig. 2C). The presence of HGF/SF elevated cell motility (columns 1 vs. 2; $p=2.0 \times 10^{-5}$). When ephrin-A1-Fc was added to the lower chamber, EphA2 activation significantly reduced the number of cells migrated to the underside of membrane (columns 2 vs. 3; $p=5.1 \times 10^{-4}$).

Because both Eph receptors and ephrin ligands are membrane-bound, Eph/ephrin interactions *in vivo* take place upon cell-cell contact. To simulate this condition, a recently described SOIL assay¹⁶ was carried out to test whether the immobilized ephrin-A1 ligand was able to inhibit chemokinetic cell migration. DU-145 cells grown to near confluence on cover slips were transferred to plates pre-coated with either Fc or ephrin-A1-Fc. HGF/SF or equal volumes of PBS control was added to the culture medium to induce chemokinetic cell migration. Fig. 2D shows that immobilized ephrin-A1-Fc reduced basal levels of cell scattering and migration off cover slips. When HGF/SF was applied, cell migration was increased. However, the increase in cell migration induced by HGF/SF was significantly reduced on the ephrin-A1-Fc coated surface.

When tumors metastasize, they may disseminate as individual cells or as a group of cells (chain migration). In the latter case, the characteristics of cell-cell adhesion are still present²⁹. To test if EphA2 activation inhibited chain migration, a scratch-wounding assay was carried out

(Fig. 2E). A wound was generated on confluent DU-145 cells using a micropipette tip. In comparison with Fc, ephrin-A1-Fc treatment slowed down the speed of wound closing. When ephrin-A1-Fc was used together with HGF/SF, the wound closing was also delayed compared with cells treated HGF/SF and Fc. This again demonstrated the inhibitory effect of EphA2 activation on cell motility. In sum, EphA2 activation potentially antagonizes the stimulatory effects of HGF/SF on four different modes of cell motility.

EphA2 activation suppressed HGF/SF-induced invasion through MatriGel

In addition to increased cell motility, the ability to invade through extracellular matrix is another characteristic of metastatic tumor cells. Therefore, we next examined the consequence of EphA activation on invasive ability of DU-145 cells induced by HGF/SF. The assay system is similar to Boyden chamber used in the cell migration assay, but the membranes were coated with growth factor-reduced MatriGel. Twenty-four hours after plating, cells invading through MatriGel and migrating to the underside of the membranes were counted (Fig. 3). The presence of ephrin-A1-Fc in the lower chamber marginally reduced the basal cell invasion (columns 1 vs. 2; $p=0.06$). Addition of HGF/SF to the lower chamber significantly increased chemotactic cell invasion (columns 1 vs. 3; $p=0.001$). When ephrin-A1-Fc was added together with HGF/SF in the lower chamber, the number of cells invading through MatriGel was significantly reduced (columns 3 vs. 4; $p=2 \times 10^{-4}$). Therefore, activation of EphA receptor has negative effects on malignant prostate cancer cell invasion.

EphA2 activation inhibited DU-145 cell scattering induced by HGF/SF and preserved E-cadherin mediated cell-cell adhesion

Tumor cell scattering and detachment from primary tumors is considered as the first step in tumor metastasis. In addition to promoting cell migration and invasion, HGF/SF is known to

disrupt cell-cell adhesion mediated by E-cadherin, induce epithelial-mesenchymal transition (EMT) and cause cell scattering³⁰. Therefore, we examined the effects of EphA2 activation on EMT and scattering induced by HGF/SF. DU-145 cells were treated with Fc (control) or ephrin-A1-Fc in the presence or absence of HGF/SF. Cell morphology was recorded 24 and 48 hours after the addition of stimuli (Fig. 4A and B). In control Fc-treated cells, only about 5% of cells were found as individual cells with no direct contact with neighboring cells, while remaining cells aggregate to form colonies with typical epithelial morphology. Ephrin-A1-Fc treatment consistently induced more compact cobblestone morphology (Fig.4A). Following 24-hour HGF treatment, most of cells underwent EMT characterized by detachment from each other and transformation into spindle-shaped fibroblastic morphology. However, in the presence of ephrin-A1-Fc cell-cell contact was significantly retained; the fraction of scattered cells was reduced from 95% to 15% accompanied by a more rounded morphology compared with cells treated HGF and Fc. This change of morphology persisted for 48 hours (Fig.4A right panel and Fig. 4B lower panel). Thus ephrin-A1-Fc significantly suppressed HGF-induced EMT in DU-145 cells.

It has been reported that HGF/SF induces E-cadherin degradation in DU-145 cells following 24 hours of treatment or longer, which in turn contributes to its cell scattering effect³¹. We next examined the changes in E-cadherin expression in cells stimulated with HGF/SF, ephrin-A1-Fc or both. Ephrin-A1-Fc treatment moderately increased E-cadherin expression (Fig. 5A, lanes 1 vs. 2 and 5 vs. 6). In agreement with previous reports³¹, HGF/SF treated cells lost most E-cadherin (lanes 3 and 7). When HGF/SF and ephrin-A1-Fc were added together, E-cadherin was partially preserved when compared with HGF/SF treated cells (lanes 3 vs. 4 and 7 vs. 8). Changes in β -catenin expression followed the same pattern as that of E-cadherin (not shown), suggesting that the E-cad-catenin complex was targeted for degradation.

To further verify this result, an immunofluorescence staining was carried out. As shown in Fig. 5B, E-cadherin resided at cell-cell junction in Fc and ephrin-A1-Fc treated cells as expected. In HGF/SF treated cells, E-cadherin staining showed largely cytosolic pattern at 24 hours, which persisted to 48 hours. Note that in some HGF/SF-treated cells, E-cadherin disappeared from the cell-cell junctions even in those cells that remained juxtaposed to each other. When ephrin-A1-Fc was added together with HGF/SF, E-cadherin expression at cell-cell junctions was dramatically retained in comparison with HGF/SF/Fc treated cells. This contrasts with immunoblot results (Fig. 5A), where only a moderate increase in E-cadherin level was detected. Therefore, while HGF/SF/ephrin-A1-Fc cotreatment moderately prevents the loss of total cellular E-cadherin, it dramatically preserves the localization of E-cadherin at cell-cell adhesion sites. RT-PCR analyses did not detect significant changes in E-cadherin transcription; nor was there a substantial variation in protease activity as revealed by zymogram analysis of cells or the secreted media between HGF- vs HGF plus ephrin-A1-treated groups (not shown). However, since total levels of E-cadherin only exhibited moderate elevation during the 24-48 hour treatment, it remains possible that a subtle changes in E-cadherin transcription and degradation may be lead to the observed increase.

To test if ephrin-A1 can serve as a preventative measure to reduce cell scattering and EMT induced by HGF/SF, we pretreated DU-145 cells with Fc or ephrin-A1-Fc for 24 hours before HGF/SF was added for additional 24 hours. At the end of treatment, immunoblot (Fig. 5C) was carried out to detect E-cadherin expression. Ephrin-A1-Fc treatment increased E-cadherin expression at 48 hours in comparison with Fc treatment (lanes 2 vs. 3), whereas HGF/SF decreased E-cadherin expression (lanes 2 vs. 5). More importantly, pretreatment with ephrin-A1-Fc significantly prevented HGF/SF-induced down-regulation of total cellular E-cadherin (Fig.

5C, lanes 5 vs. 6), to a greater extent than cells exposed to both ephrin-A1 and HGF/SF at the same time (Fig. 5A, lanes 3 and 4, or 7 and 8). Similar to cells co-treated with HGF and ephrin-A1-Fc (Fig. 5B), immunofluorescence staining revealed that significant fraction of E-cadherin was retained at cell-cell junction (Fig. 5D). Our results indicate that EphA2 activation can be used as a preventive measure to reduce metastasis by preserving E-cadherin expression and inhibiting EMT.

DISCUSSION

In this study, we show that EphA2 is the primary EphA kinase expressed in human and rat prostate cancer cells. Moreover, the expression of EphA2, but not that of EphA1, EphA3 and EphA4, was elevated in cell lines derived from distal metastases of prostate cancer. Expression of ephrin-A ligands, on the other hand, remained low and relatively constant as prostate cancer became more malignant, accounting for the low basal activation status of EphA2 overexpressed on metastatic prostate cancer cells. More importantly, activation of the EphA2 significantly counteracted HGF/SF-induced cell scattering, migration and invasion in DU145 cells. We further demonstrated that stabilization of E-cadherin expression at adherens junction was one of the possible mechanisms contributing to the inhibitory effects of EphA2. The results indicate that the overexpressed and unoccupied EphA2 receptor on metastatic prostate cancer cells may be used as a target in malignant prostate cancer intervention.

HGF/SF was originally discovered for its ability to induce growth of hepatocytes³² and dissociation and migration of epithelial cells³⁰. Both mitogenic activity and scattering effect result from HGF/SF activating receptor tyrosine kinase c-Met. The dual activities of c-Met are crucial for cell dissociation from primary tumor as well as degradation of and invasion through

surrounding extracellular matrix. In addition, they promote adhesion, migration and growth of tumor cells at distal metastatic sites ^{33,34}. Overexpression of c-Met in multiple human cancers further demonstrates its significance in cancer progression ³⁵⁻³⁷. In prostate cancer, the overexpression of c-Met has been linked to high grade prostate cancer ³⁴ and bone metastasis ⁶. Our results indicate a parallel expression pattern of EphA2 and c-Met in human prostate cancer cell lines, low in LNCaP and CWR22R and high in DU-145 and PC-3 (data not shown). Despite similar expression pattern in the course of prostate cancer progression, EphA2 and c-Met cause opposite effects on Ras/ERK signaling pathway when activated by their respective ligands. Activation of c-Met is known to activate Ras/ERK signaling pathway ³⁸, while our results showed EphA2 activation inhibited ERK activity. Ras/ERK is one of the major signaling pathways in response to mitogenic stimuli ³⁹. We have previously reported that activation of EphA2 could inhibit proliferation of several cell types including PC-3 prostate cancer cells ²⁶. However, because the inhibitory effects of ephrin-A1 on ERK1/2 were transient, they are unlikely to be a direct mechanism responsible for the prolonged inhibitory effects of EphA2 ligation on cell migration, invasion and scattering. However, as discussed below, the transient effects may have an indirect role by triggering other cellular changes.

EphA2 and E-cadherin co-localized at intercellular junctions in MCF-10A human breast epithelial cells ⁴⁰ as well as MDCK cells ¹⁶. The results reported here showed that EphA2 is also localized to cell-cell adhesion sites in DU-145 cells, and EphA2 activation reduced the loss of E-cadherin induced by HGF/SF. E-cadherin is one of the key molecules involved in homophilic cell-cell interactions of epithelial cells. Decreased E-cadherin expression was linked to the progression of prostate cancer ^{41,42}. Gene mutation ⁴³ or promoter methylation ⁴⁴ often accounts for the diminished E-cadherin expression in cancer cells. However, previous reports have shown

that increased degradation of E-cadherin is a major mechanism responsible for its loss from adherens junctions upon HGF/SF treatment of LNCaP and DU-145 human prostate cancer cell lines. Moreover, matrilysin (MMP7) activity is found to be the major protease mediating the cleavage at E-cadherin extracellular domain ^{45 46}.

Interestingly, emerging evidence demonstrates the involvement of ERK1/2 in HGF/SF-induced MMP up-regulation. Tanimura et al. ⁴⁷ showed that HGF/SF-induced ERK activation resulted in elevated MMP-2 and -9 expression and, consequently, increased cell motility of MDCK cells. In addition, ERK1/2 activation is required for HGF/SF induced adherens junction disassembly in MDCK cells ⁴⁸. Although there is no direct evidence demonstrating the involvement of Eph receptor activation on MMP activity thus far, we showed that EphA2 activation down regulated ERK activities. Whether this transient inhibition of ERK activity is sufficient to counteract the HGF/SF effect on MMP up-regulation is presently unclear, although our long term studies following 24 to 48 hour stimulation did not reveal significant changes in protease activity on zymogram analysis. However, because the change in total E-cadherin levels was relatively small, it remains possible that even a slight alteration in protease activity may lead to the observed decrease during overnight treatment. Further investigation is needed to determine if this or other mechanisms may be implicated in retaining E-cadherin at cell-cell adhesion site.

There are several explanations for EphA2 overexpression during malignant progression of prostate cancer remains. One possibility is that the overexpressed and unligated EphA2 may have signaling capacities distinct from the ligated EphA2, and may promote tumor progression. For example, EphA2 may sequester signaling and adaptor proteins that in the absence of ligand may promote cell growth or migration. It has been previously reported that overexpression of EphA2 in MCF10A breast epithelial cell line causes phenotypic transformation of these cells ⁴⁹.

However, overexpression of EphA2 in CWR22R and LNCaP cells has failed to promote either the growth or migration of these cells (not shown), indicating possible cell type-specificity of EphA2 function. Alternatively, EphA2 overexpression may simply reflect general deregulation of gene expression machinery during tumor progression. Although more studies are necessary to distinguish between these possibilities, the fact that activation of EphA2 suppresses Ras/ERK signaling cascade and counteracts behaviors associated with malignant progression suggest EphA2 may be exploited to slow prostate cancer progression. In this regard, we show here that while EphA2 expression is increased as prostate cancer progress, the ligand expression remained relatively constant and low across all the cell lines examined, consistent with the low EphA2 activation level prior to the addition of ligand. Thus most EphA2 receptor on malignant prostate cancer cells is unoccupied and is available for ligand or antibody targeting.

In summary, we have shown that the activation of EphA2 was able to antagonize the HGF/SF-induced prostate cancer cell chemotaxis, matrix invasion and scattering. Preservation of E-cadherin-mediated cell-cell adhesion represents a possible mechanism for some of the effects. Since loss of cell-cell adhesion is a hallmark of malignant prostate cancer, EphA2 may be targeted to suppress prostate cancer progression promoted by the paracrine/autocrine growth factors. EphA2 may be exploited as a novel target to control the malignant progression of prostate cancer.

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Figure Legends:

Fig. 1. (A) EphA2 is the primary EphA kinase expressed on human prostate cancer cells. Cells were surface-labeled with membrane impermeable biotin and lysed (Method). EphA2 was depleted from total cell lysates with three rounds of immunoprecipitation with an EphA2-specific antibody (lower panel). "Mock" depletion with normal IgG was performed on a separate set of lysates, and was used as control (top panel). The EphA2- or mock-depleted lysates were subject to precipitation with saturating amount of ephrin-A1-Fc (5 μ g) which should bind to most members of EphA subfamily kinases including EphA2. The precipitated material was then blotted with HRP-conjugated avidin. (B) Expression of EphA2, but not other EphA kinases and ephrin-As, is elevated in cell lines derived from more malignant prostate cancer. Cells were lysed on plates and subject to immunoblot with the indicated antibodies. (C) Immunofluorescence staining of EphA2. Human prostate cancer cells grown on cover slip were stained with anti-EphA2 antibody. Texas-Red labeled secondary was used to visualize the bound primary antibody. (D) DU-145 and MatLyLu cells were stimulated with ephrin-A1-Fc for indicated times and lysed. EphA2 immunoprecipitation was carried out to determine the activation status of EphA2 using anti-phosphotyrosine antibody. The same membranes were stripped and re-blotted for total EphA2. To determine the ERK activation status, total cell lysates were analyzed by immunoblot using anti-phospho ERK (p-ERK) antibody. Same membranes were stripped and blotted with total ERK.

Fig. 2. EphA activation inhibited DU-145 cell migration. (A) Expression of c-Met is elevated in cell lines derived from more malignant prostate cancer. Total cellular proteins were

immunoblotted with anti-human c-Met. An anti-tubulin blot was used as a control for protein loading. (B) In a modified Boyden chamber assay, DU-145 cells were placed in the upper chamber and allowed to migrate to the lower chamber containing indicated reagents. After 8 hours, cells were stained with crystal violet and cells remaining in the upper chamber were removed. Top panel: Cells migrated to the undersides of membrane were counted; six high power fields were counted. Results represent means \pm S.D. Representative fields were shown in the lower panel. (C) Ephrin-A1-Fc inhibits DU-145 cell migration when HGF/SF was uniformly presented in both sides of membranes. The experiment was carried out as described in A. (D) Inhibition of chemokinetic cell migration by immobilized ephrin-A1 in a SOIL assay (Method). Ephrin-A1-Fc or control Fc was immobilized on 6-well culture dishes. Freshly confluent DU-145 cells on cover slips were transferred to the coated surfaces. Cells were stimulated with HGF/SF to induce migration off the cover slips to immobilized Fc or ephrin-A1-Fc. After 48 hours, cells were fixed and stained with crystal violet. The cells that had migrated to the coated surface were photographed after removing cover slips. (E) DU-145 cells were grown to confluency and a wound was generated by scratching monolayer with a 200 μ l pipette tip. The wound was recorded prior to and 12 hours after the addition of Fc, ephrin-A1-Fc (1 μ g/ml) and/or HGF/SF (20 ng/ml) at the marked positions. Representative results from 3 independent experiments are shown.

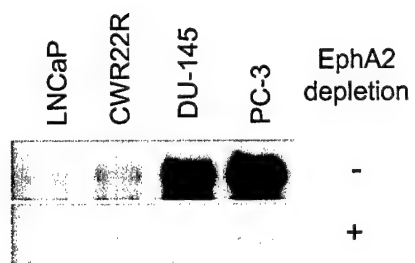
Fig. 3. EphA activation inhibited DU-145 cell invasion. DU-145 cells were placed in the upper chamber of MatriGel-coated Transwell. Fc, ephrin-A1-Fc, HGF/SF/Fc or HGF/SF/ephrin-A1-Fc, were added in the lower chamber. After 24 hours, cells invading through MatriGel and migrated

to the undersides of membrane were counted. Representative results (mean \pm S.D.) from three independent experiments are shown.

Fig. 4. EphA activation attenuated cell scattering induced by HGF/SF. DU-145 cells were plated on 6-well dishes at 1×10^5 cells/well and cultured in the presence of indicated reagents. Cell morphology was recorded 24 and 48 hours after the initiation of treatments (A). Numbers of total cells and cells without contact with other cells were counted in 6-8 randomly chosen fields (B). * HGF/SF/ Fc vs. HGF/SF/ephrinA1-Fc: $p < 10^{-5}$.

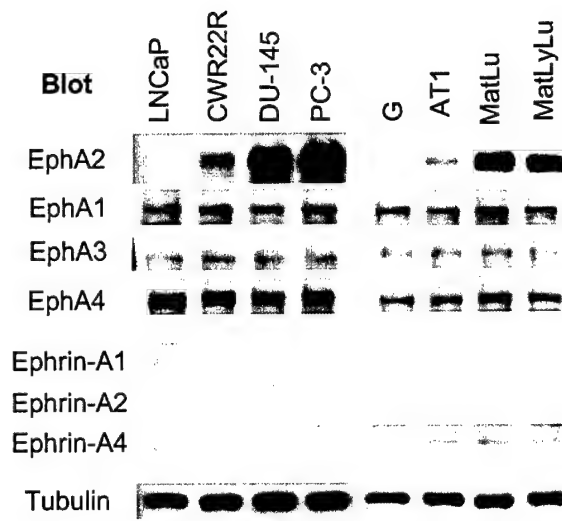
Fig. 5. (A) Ephrin-A1 co-stimulation inhibited the loss of E-cadherin induced by HGF/SF. DU-145 cells were treated as indicated for 24 or 48 hours. Cell lysates were subject to anti-E-cadherin immunoblot. Same membrane was stripped and blotted with anti-ERK1 as loading control. Relative intensity of E-cadherin = intensities of E-cadherin band / intensity of ERK band. (B) Immunofluorescent staining of E-cadherin after 24 hours of the indicated treatment. Note almost exclusive cytoplasmic staining pattern in HGF/SF treated cells, while cotreatment with ephrin-A1 restored significant levels of E-cadherin at cell-cell junction. (C) Ephrin-A1-Fc pre-treatment preserved E-cadherin expression in HGF/SF treated cells. DU-145 cells were pre-treated with Fc or ephrin-A1-Fc for 24 hours. HGF/SF was then added. The cells were cultured for another 24 hours and lysed for anti-E-cadherin immunoblot. Anti-ERK1 was used as loading control. (D) Cells grown on cover slips received same treatment as in C were subject to anti-E-cadherin immunofluorescence analysis. Note the significant preservation of E-cadherin localization at cell-cell adhesion site by ephrin-A1-Fc compared with Fc control.

A



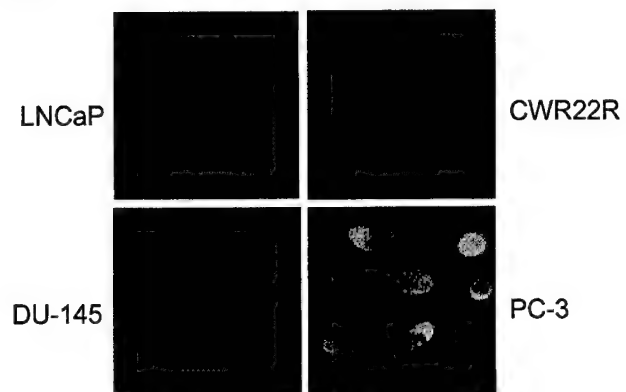
Wei et al. Figure 1A

B



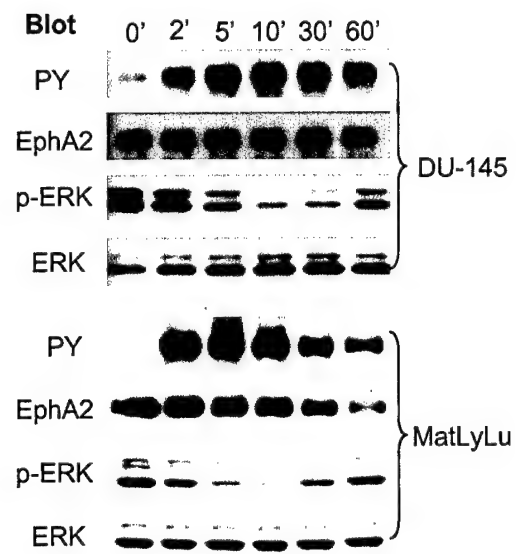
Wei et al. Figure 1B

C



Wei et al. Figure 1C

D

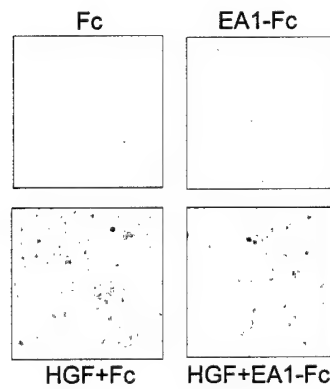
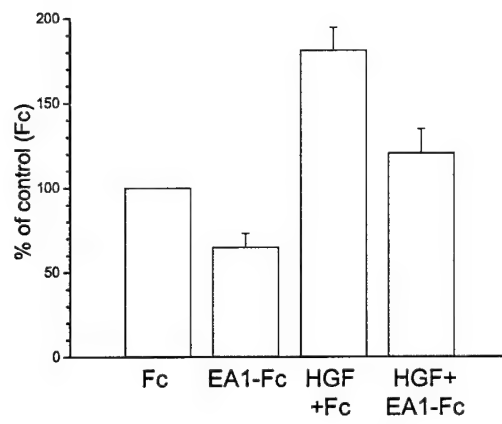


Wei et al. Figure 1D

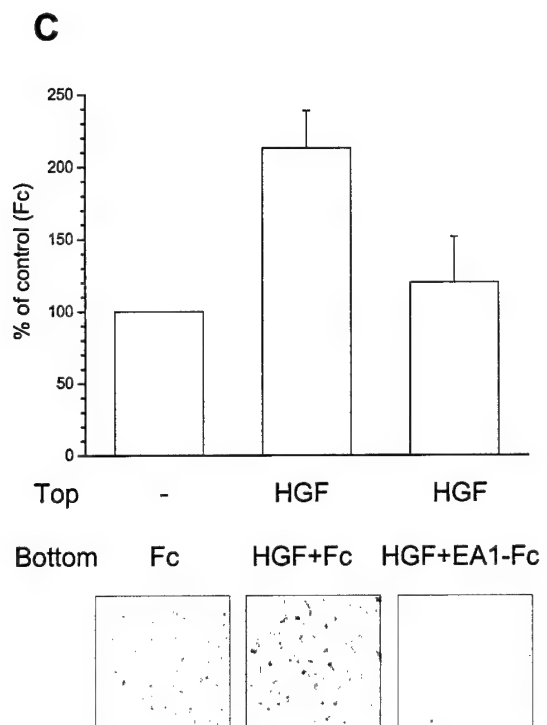


Wei et al. Figure 2A

B

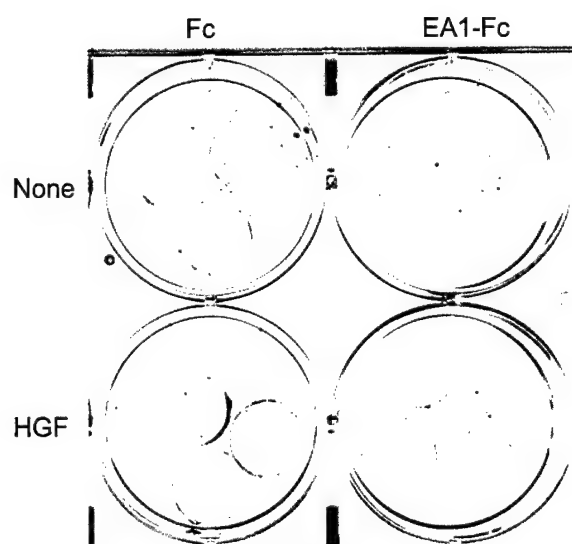


Wei et al. Figure 2B

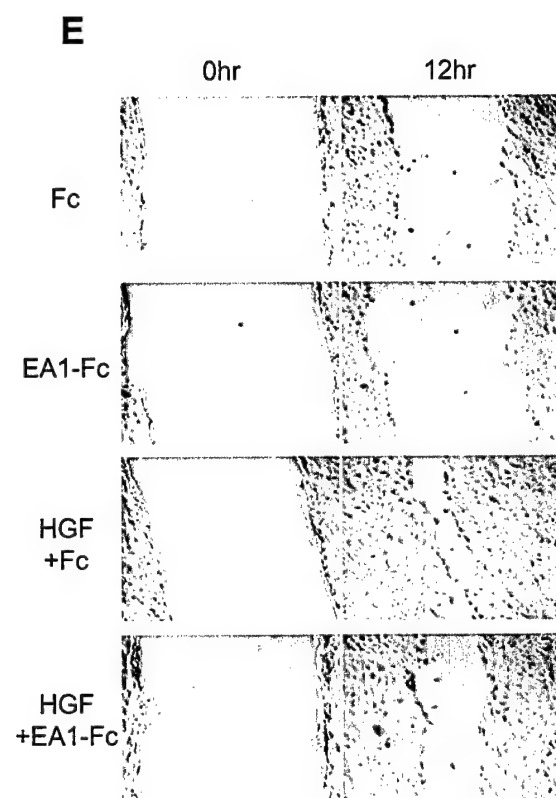


Wei et al Figure 2C

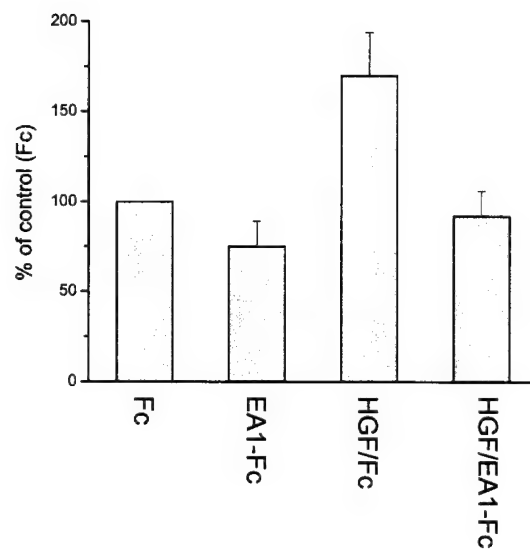
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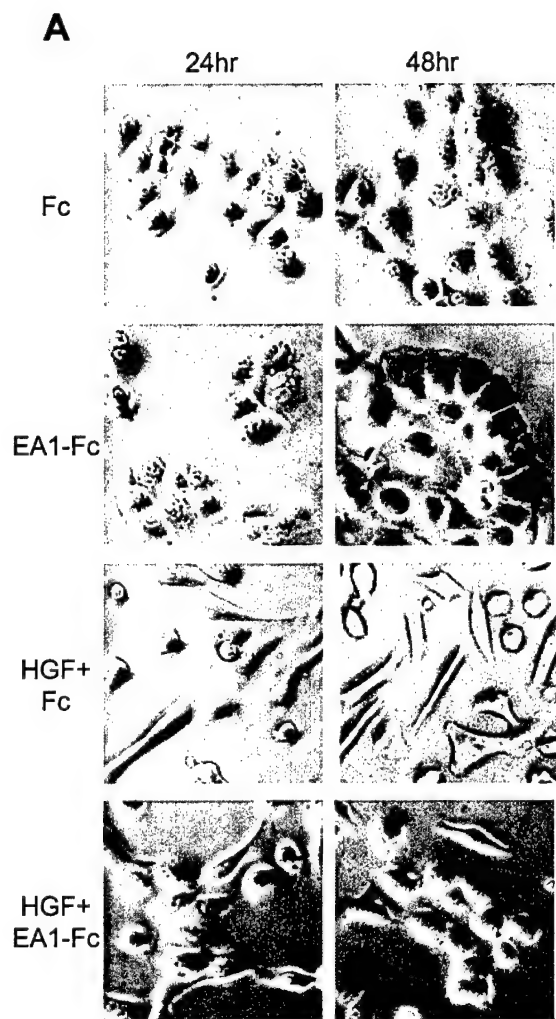
Wei et al. Figure 2D



Wei et al Figure 2E

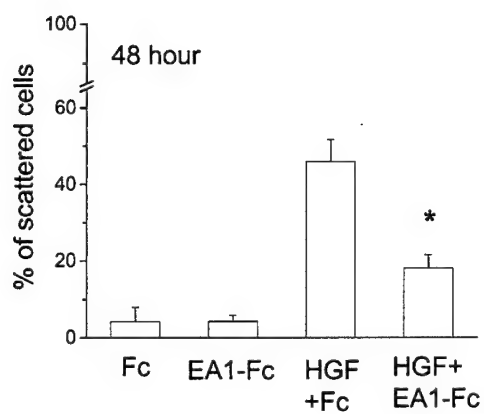
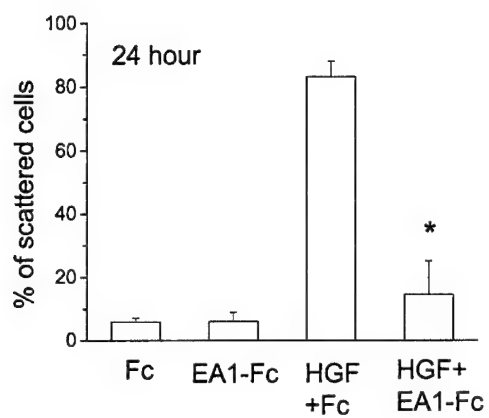


Wei et al Figure 3

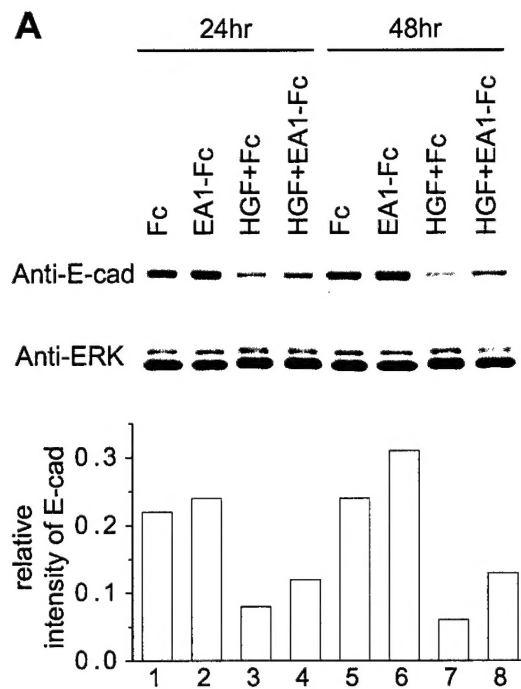


Wei et al Figure 4A

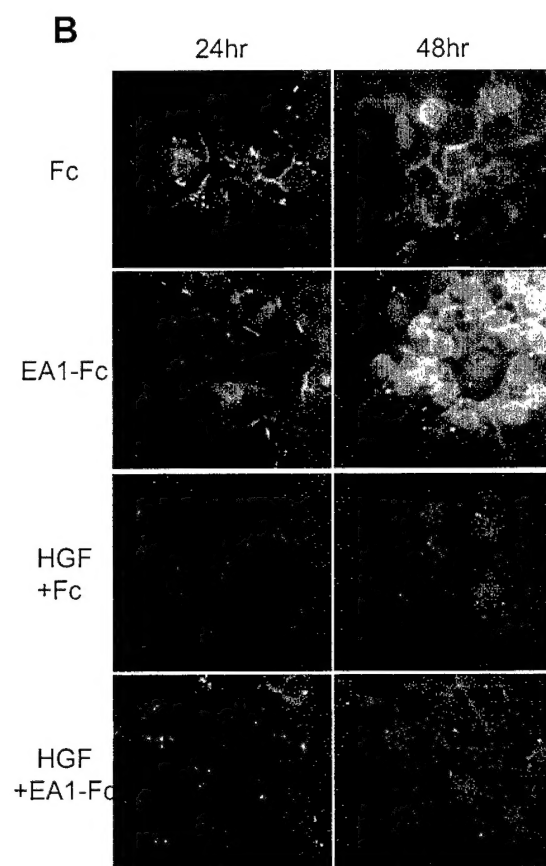
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Wei et al. Figure 4B

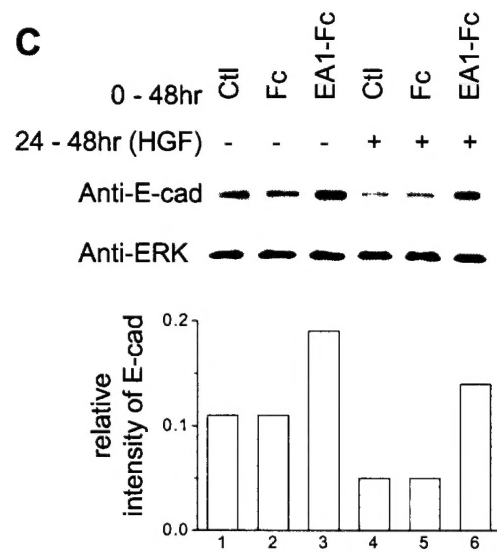


Wei et al Figure 5A



Wei et al. Figure 5B

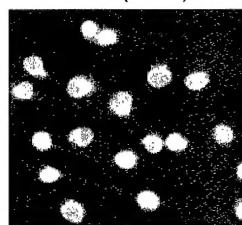
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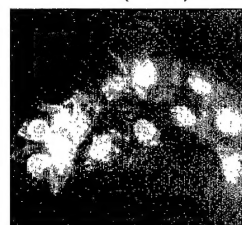
Wei et al. Figure 5C

D

Fc (48hr)
HGF(24hr)



EA1-Fc (48hr)
HGF(24hr)



Wei et.al. Figure 5D

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